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Full Length Research Paper

Biochemical characterization of *Fusarium oxysporum* f. sp. *cubense* isolates from India

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The Fusarium wilt caused by *Fusarium oxyspoum* f. sp. *cubense* (Foc) is a major biotic constraint for banana production. The characteristics of *F. oxyspoum* f. sp. *cubense* isolates were investigated using electrophoretic studies of isozyme and whole-cell protein. The morphological characteristics of the isolates were very similar to each other. All the Foc isolates were pathogenic to banana cultivar 'Nanjangud Rasabale' but they did not induce any disease symptoms on cultivar 'Cavendish'. *F. oxyspoum* (Isolate 6) did not induce wilt symptoms on either 'Nanjangud' or 'Cavendish' cultivar. Isozyme banding patterns showed 46 scoreable markers and cluster analysis with UPGMA using genetic distance showed that the isolates belonged to three main groups. Group 1 contained isolates 1, 2, 4, 5, 7 and isolate 3 and 6 were placed in group 2 and 3. Results indicated that the estimated intra-specific variation may be more pronounced with isozyme analysis than with protein markers. The level of isozyme variability detected within *F. oxysporum* f.sp*. cubense* suggested that it is reliable, efficient and effective in determining genetic relationships among Foc isolates.

Key words: Fusarium wilt, *Fusarium oxysporum* f. sp. *cubense*, polyacrylamide gel electrophoresis, isozyme analysis.

INTRODUCTION

Banana (*Musa* spp.) is a major fruit of India, which accounts for about 32% of the total fruit production. The 'Nanjangud rasabale' (NRB) is an elite native variety of banana originated from a place called 'Nanjangud' in Karnataka State. It is popular and highly priced fruit in Karnataka and in other states because of its special qualities such as taste, aroma, color, fibrous texture and nutritional content. It has a triploid (AAB) genome and the hybrid origin belongs to *Musa paradisiaca.* The wilt of banana, caused by *Fusarium oxysporum* f.sp. *cubense* (Smith, 1910; Snyder and Hansen, 1940), especially race 1, is an important disease on Rasthali group (AAB) of banana. The assessment of genetic diversity of Foc is required for the development of long-term disease management strategies.

Different types of biochemical markers are available for use in evolutionary and population studies of phytopathogenic fungi (Michelmore and Hulbert, 1987; Tudzynski and Weltring, 1993). Electrophoretic analysis of whole cell proteins by one-dimensional protein patterns provides a rough measure of the number and physicochemical properties of gene products. One-dimensional polyacrylamide gel electrophoresis of proteins has been used extensively for identification and classification at the strain and species level (Snider, 1973). Methods like isozyme analysis on starch or polyacrylamide electrophoresis provides a well-established and efficient tool for revealing genetic variability in fungal populations (Micales et al., 1986). The protein polymorphism reflects the genetic background of the microorganisms (Shaw, 1965). The expression of alleles coding for different isozymes

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Abbreviations: UPGMA, Unweighted pair-groups method using arithmetic mean; **NRN,** 'nanjangud rasabale'; **PDA,** potato dextrose agar; **PDB,** potato dextrose broth; **EDTA,** ethylene diamine tetraacetic acid; **POX,** peroxidase; **SOD,** superoxide dismutase; **GDH,** galactose dehydrogenase; **G,** β- 1,3-glucanase; **SDH,** Succinate dehydrogenase; **CAT,** Catalase; **VCG,** vegetative compatibility group; **SDS-PAGE,** sodium dodecyl sulfate-polyacrylamide gel electrophoresis; **DNA,** deoxyribonucleic acid; **RAPD,** random amplified polymorphic DNA; **IGS,** intergenic sequence.

are more or less independent from the environment; thus, this approach offers a relatively neutral means of determining genetic variation. In spite of this advantage, the application of isozyme techniques in studies of phytopathogenic fungi has been limited until now. The main reason of the limited use of isozymes could be the low level of polymorphism found in various fungal taxa examined (Burdon, 1993). Nevertheless, this approach was successfully applied in *Rhizoctonia solani* (Liu and Sinclair, 1992), *Fusarium* spp. (Aly et al., 2003) and *Phytophthora infestans* (Tooley et al., 1985) to assist in population studies, to distinguish isolates of *Peronosclerospora* (Bonde et al., 1984) or *Aphanomyces* species (Larsson, 1994) and to reveal the probable center of origin of *Phytophthora citrophthora* (Mchau and Coffey, 1994).

Isozyme analysis is considered to be a relatively economical and practical technique for screening large populations and identifying species (Guarro et al., 1999) and has been widely used in bacterial and fungal taxonomy as well as other organisms to study the diversity at interspecific or intraspecific levels in a population (Carlile and Watkinson, 1994). Bosland and Williams (1987) have used isozymes to differentiate *Fusarium* species and four *F. oxysporum* formae speciales. Laday and Szecsi (2001) indicated that isozyme data provided an excellent diagnostic tool for the identification of *Fusarium* spp. Variations among five *Fusarium* spp. were analyzed by 12 morphological characters and 78 isozymic bands by Luo et al. (2007). Isozyme patterns showed higher varieties between species and among individual isolates than morphological characters. Studies of intra- and extracellular isozyme banding patterns (esterase, superoxide dehydrogenase, malate dehydrogenase, dihydrolipoamide dehydrogenase and succinate dehydrogenase) of *Fusarium oxysporum* has been successfully employed for genetic variability analysis of *F. solani* isolates of guava wilt (Skovgaard and Rosendahl, 1988; Gupta et al., 2009). Electrophoretic detection of protein banding patterns and esterase isozymes in polyacrylamide gel electrophoresis were used for the identification of some *Fusarium* isolates (El-Kazzaz et al., 2008). It was found that electrophoretic analysis of esterase is a useful tool for differentiating between *Fusarium* spp. as well as different formae speciales of *F. oxysporum* (Aly et al., 2003).

The objective of the present study was to estimate the diversity of Foc isolates collected from different geographical regions of India based on isozyme analysis and protein gel electrophoresis. This information is important in screening host resistance to Fusarium wilt and monitoring shift in virulence.

MATERIALS AND METHODS

Collection of Foc Isolates and their maintenance

Foc isolates were obtained from National research institutions namely Bhabha Atomic Research Center (BARC) (Isolate No. 3), Tamilnadu Agricultural University (TNAU) (Isolate No. 4), National Research Center on Banana (NRCB) (Isolate No. 5) and Indian Agricultural research institute (IARI) (Isolate No. 7). Isolate No. 2 was isolated and maintained in the Department of Applied Botany, University of Mysore, Mysore. An isolate of Foc (Isolate No. 1) was from the corm pieces of wilt-affected 'NRB' plants 'Devarasanahalli' near 'Nanjangud'. The fungus was isolated by plating surface-sterilized corm pieces on PDA. A non-virulent *F. oxysporum* isolate No. 6 was isolated from soil of banana plots. A total of seven isolates were collected from different sources and were numbered serially.

Pathogenicity Test

Foc isolates were tested for their pathogenicity on 'NRB' and 'Cavendish' plantlets (4 replicates of 5 plantlets each). Tissue culture plants that were ready for hardening were used for the experiment and tested by the procedure described by (Liew, 1996). Two sizes of plastic cups were selected. Basal cup was slightly wider and fitted into the upper cup. The upper cup was punctured at the base. Sterilised sand was partially filled into the upper cup. Banana plantlets were placed individually in the upper cup. Hoagland's nutrient solution (Hoagland and Amon, 1950) was poured to the sand. Plantlets were kept in the growth chamber with high humidity for 3 - 4 weeks. One-week-old culture of Foc was taken and the spore suspension was adjusted to 5 x 10^5spores/ml using a haemocytometer. The plants were removed carefully so as not to damage the root system and immersed into a container of sterile water and then into the spore suspension for 15 min. The inoculated plantlets were then replaced in to the same cup. The inoculated plants were kept inside the growth chamber.

Growth conditions and preparation of crude protein

The isolates of Foc were grown on PDB at 25 ± 2°C in 500 ml Erlenmeyer flasks on a shaker at 200 rpm for 4 days. Mycelia were collected by filtration through filter paper and washed twice with cold sterile distilled water. After squeezing dry, harvested mycelia were stored at –80°C until used.

Frozen mycelia (2.5 g) were ground into fine powdered using ice-cold buffer composed of 50 mM phosphate buffer pH 7.2 + 50 mM EDTA in a mortar and pestle in the presence of 1 g of sterile sand and transferred to centrifuge tubes. The mixture was centrifuged at 10,000 g for 20 min at 4°C and the supernatant was collected. The protein content was estimated by dye binding method (Bradford, 1976) using serum albumin as the standard.

Electrophoresis and enzyme staining

Proteins were separated on polyacrylamide gels according to the method of Laemmli (1970) using dual vertical slab gel electrophoresis apparatus (Amersham Pharmacia Biotech). The dimensions of the gels were 10 x 8 cm and 1.0 mm thick. The gel consisted of 12% for separating gel and 5% for stacking gel.

The protein concentration was determined using spectrophotometer (Hitachi U2000, Japan) and 30 µl equivalent of each protein sample was mixed with equal volume of sample buffer and was boiled for 5 min. After clarification by low-speed centrifugation, the samples were loaded into wells in the slab gel. Standard proteins for molecular weight determination (Genei, Bangalore) were run parallel to the samples. Initial voltage of 50 V at 25 mA was provided until the samples traversed the stacking gel and after reaching the separating gel, the power supply was increased to 100 V. The samples were run till the marker dye reached the bottom of the gel plate. After electrophoresis, the protein were visualised by

Table 1. List of isozymes studied and Enzyme commission number (EC).

ENZYME	Enzyme commission (EC) number	Abbreviation
Peroxidase	1.11.1.7	POX
Superoxide dismutase	1.15.1.1	SOD
Catalase	1.11.1.6	CAT
β-1,3 Glucanase	3.2.1.39	GLU
Phosphorylase	2.4.1.1	-------
Protease	3.4.2.3	PRO
Cellulase	3.2.1.4	CEL
Galactose dehydrogenase	1.1.1.48	GDH
Succinate dehydrogenase	1.3.99.1	SDH
Glucose-6-phosphate dehydrogenase	1.1.1.49	G6DH

coomassie blue staining. The stained gels were analysed by Bioprofile Image Analysis System (Vilber Lourmat, France). The molecular weights of the stained protein were calculated using the in-built software of this system.

Isozyme analysis

Isozymes were separated using 8% separating and 5% stacking gels according to the method of Davis (1964). For detection of phosphorylase isozyme. 0.5% soluble starch was incorporated into the separating gel. After electrophoresis, the gels were stained with specific enzyme stains. The details of enzyme systems considered and abbreviations are given in Table 1.

Data analysis

To determine relatedness of the fungal isolates, the isozyme data from all the gels were recorded and compiled in a binary matrix in which 1 indicated the presence and 2 the absence of the bands. The data were converted to distance matrices based on (Priest and Austin, 1993) unbiased minimum distance. The distance matrices were then used to construct a dendrogram by the Unweighted Pair-Group Method with arithmetic mean (UPGMA) using Tools for Population Genetic Analysis (TFPGA Ver 1.3) (Miller, 1997).

RESULTS

Pathogenicity test

None of the Foc/Fo isolates infected banana cultivar 'Cavendish'. Isolates 1, 2, 3, 4, 5 and 7 induced yellowing of leaves between 40 - 45 days and discoloration of stele in rhizome in cv. 'Rasabale' while isolate 6 did not induce any reaction. This confirmed the virulence of isolates 1 to 5 and 7.

SDS-PAGE

The protein profile of different isolates showed common protein band of 53 kDa in all Foc isolates except isolate 6 and also it could be differentiated by distinct bands for instance two proteins of molecular weight 51.9 and 43.0 kDa were detected only in isolate 1. Protein bands of 27.4, 20.4, 19.3 and 16.9 kDa were observed only in isolate 3. Protein band of 109.6 kDa was present only in isolate 6 (Figure 1).

Isozyme analysis

Activity of 10 enzymes was determined in mycelial extracts of Foc/Fo. Nine of them provided clear isozyme pattern. Glucose-6-phosphate dehydrogenase activity was not found in any of the Foc isolates tested. Peroxidase, superoxide dismutase, phosphorylase, catalase, cellulase, protease, β-1,3-glucanase, succinate dehydrogenase and galactose dehydrogenase showed good resolution.

A major isoform of peroxidase (POX) was present only in isolate 3 whereas POX5 isoform was present only in isolate 1 (Figure 2). Six isoforms were observed for superoxide dismutase (SOD) with SOD1 isoform exclusively present in isolate 3. SOD4 and SOD5 isoforms were present in isolates 1, 2 and 3 (Figure 3). One isoform of phosphorylase (Figure 4) was observed in isolates 1, 4, 5 and 7 and Protease-3 isoform was present in isolate 4, 5 and 7 (Figure 5). Isolate 3 showed unique banding pattern of catalase with low molecular weight isoform CAT3, 4 and 5. CAT1 isoform was present in isolates 1, 2, 4, 5 and 7 (Figure 6). Galactose dehydrogenase showed eight isoforms with higher activity seen in isolate 1. Isoform GDH5 was present in isolates 1, 4, 5 and 7 only. Isoform GDH8 was present in avirulent isolate 6 and not in other isolates (Figure 7). Two isoforms were observed in β- 1,3-glucanase (G1 & G2) with G1 isoform present only in isolate 2 and G2 in 1, 4, 5 and 7th isolates (Figure 8). The avirulent isolate did not reveal any of the β- 1,3-glucanase isoforms. A major isoform was detected in isolate 3 and 5 for cellulase and isolate 2 did not reveal any isoform for cellulase (Figure 9). Succinate dehydrogenase isoform 5 was prominently detected in isolates 1, 4, 5 and 7 (Figure 10).

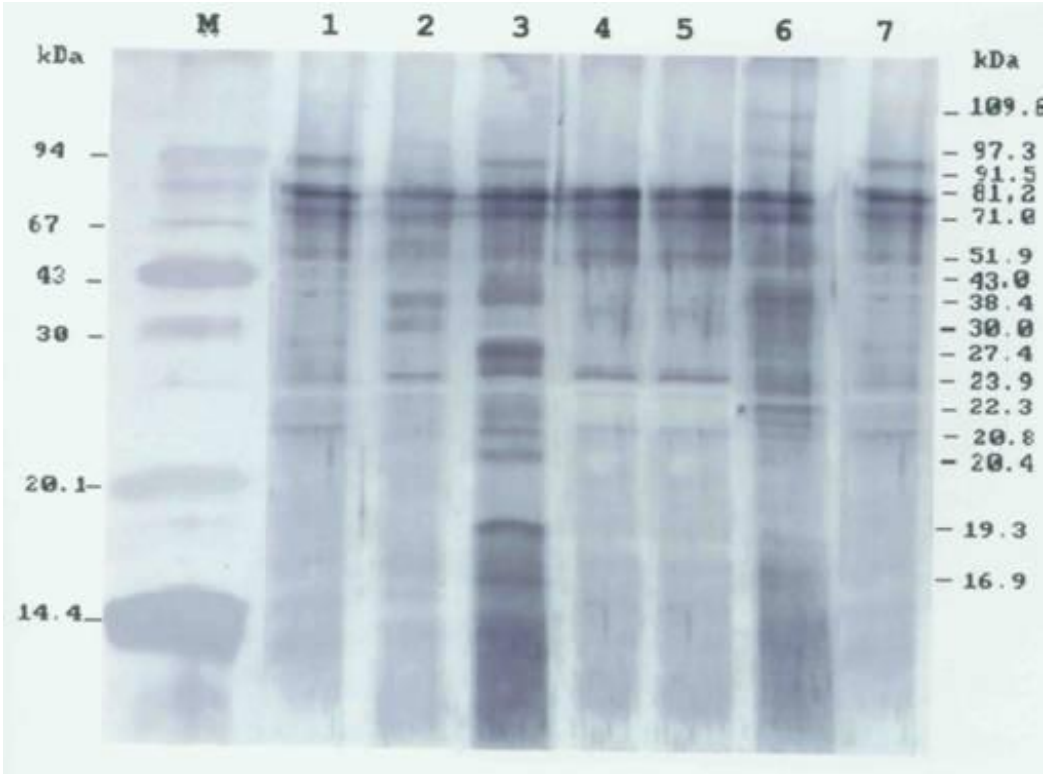

Figure 1. Protein banding pattern of six *Foc* isolates and one *Fo* isolate after SDS-polyacrylamide gel electrophoresis. M, Marker; the numbers represent the isolates.

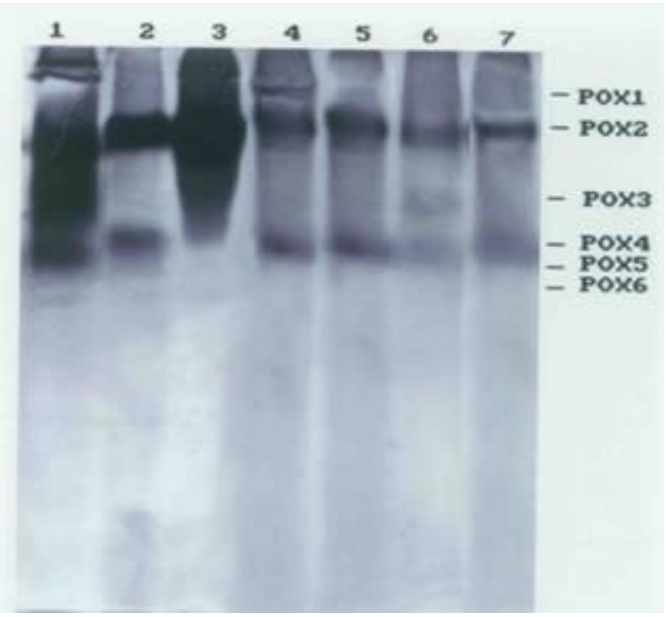

Figure 2. Peroxidase isozyme profile of *Foc* and *Fo* isolates. The numbers represent the isolates.

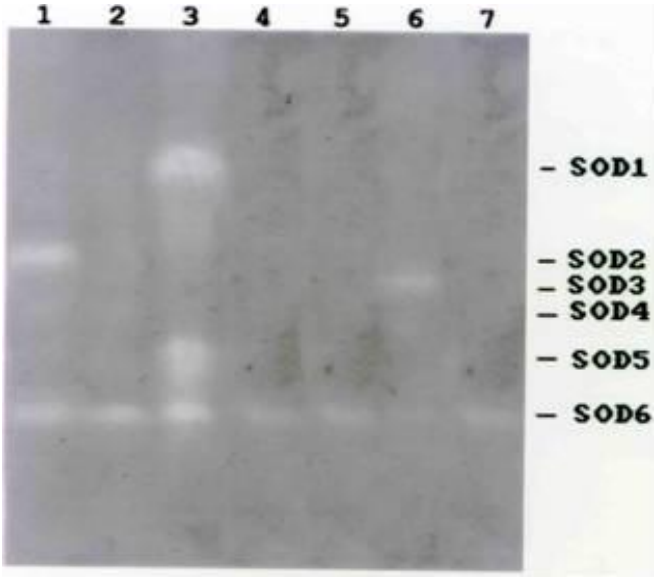

Figure 3. Superoxide dismutase isozyme profile of *Foc* and *Fo* isolates. The numbers represent the isolates.

Cluster analysis of isozyme profiles

All the enzymes assayed revealed multiple bands. A total of 46 electrophoretic phenotypes were detected among seven isolates examined. A cluster analysis with Un-weighted Pair-Group Method with arithmetic mean (UPGMA) using genetic distances showed that the

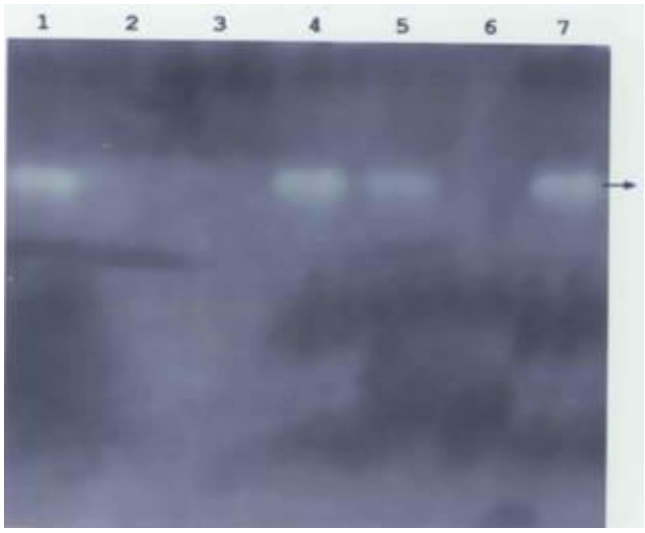

Figure 4. Phosphorylase isozyme profile of *Foc* and *Fo* isolates. The numbers represent the isolates.

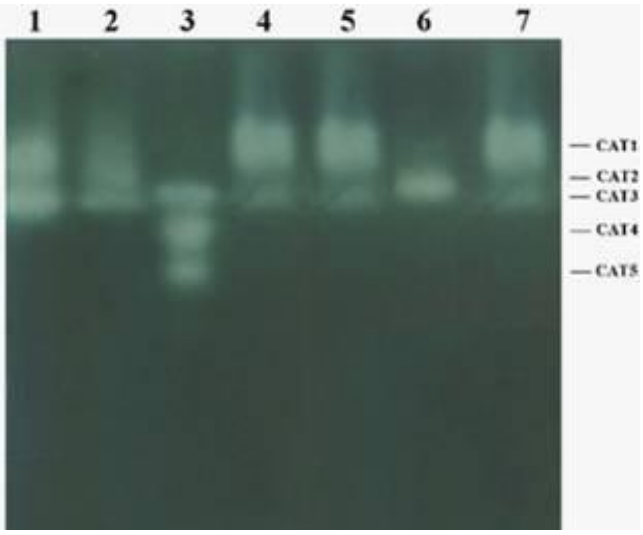

Figure 6. Catalase isozyme profile of *Foc* and *Fo* isolates. The numbers represent the isolates.

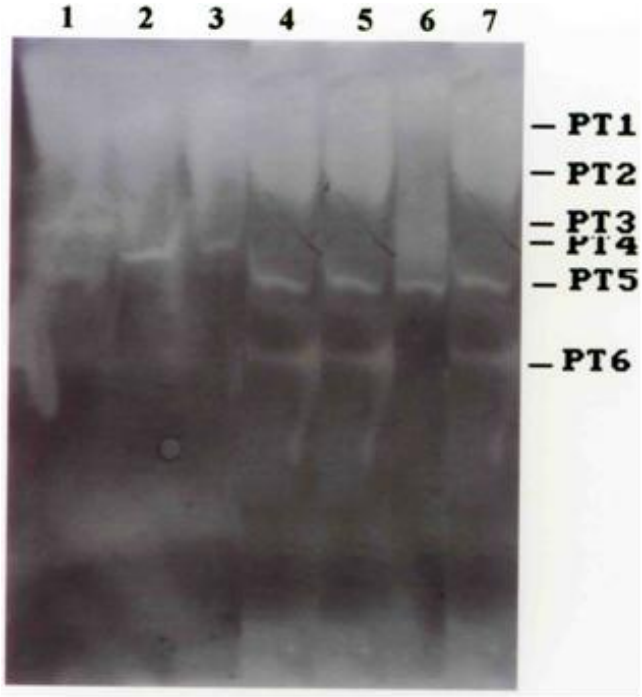

Figure 5. Protease isozyme profile of *Foc* and *Fo* isolates. The numbers represent the isolates.

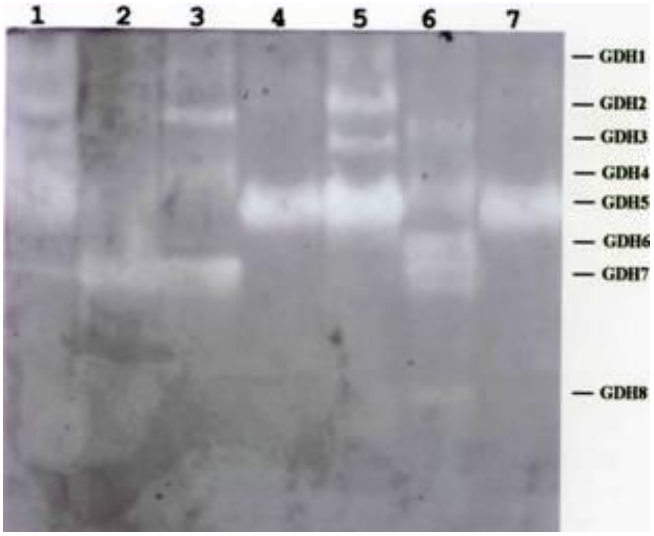

Figure 7. Galactose dehydrogenase isozyme profile of *Foc* and *Fo* isolates. The numbers represent the isolates.

isolates fell into three main groups and the first main group is divided into three sub-clusters. The first group contains isolates 4 and 5 with a distance of d = 0.185, closely related to isolates 1 and 7 with a distance of d = 0.285 while isolate 2 is near to isolate 1 and 7 with a distance of d =0.425. The second group is distinctly away from the first group with a distance of d = 0.700 represented by isolate 3. The third group is isolate 6 with a distance of d = 0.735 and 7 was closely related to isolate 3 (Figure 11).

DISCUSSION

Fusarium wilt of banana caused by Foc race 1 is a major threat for cultivation of rasathali group of banana. Breeding for resistance against this disease is difficult because of triploid genome (AAB), with A = 11 chromosomes. Genetic variation within local population of the fungus is required to delineate the races and to understand the pathogenicity spectrum of the fungus, which could enhance the effectiveness of germplasm screening and

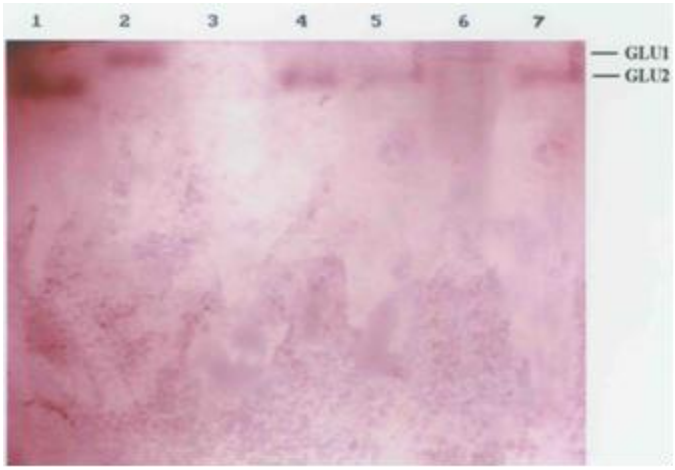

Figure 8. β-1, 3 glucanase isozyme profile of *Foc* and *Fo* isolates. The number represents the isolates.

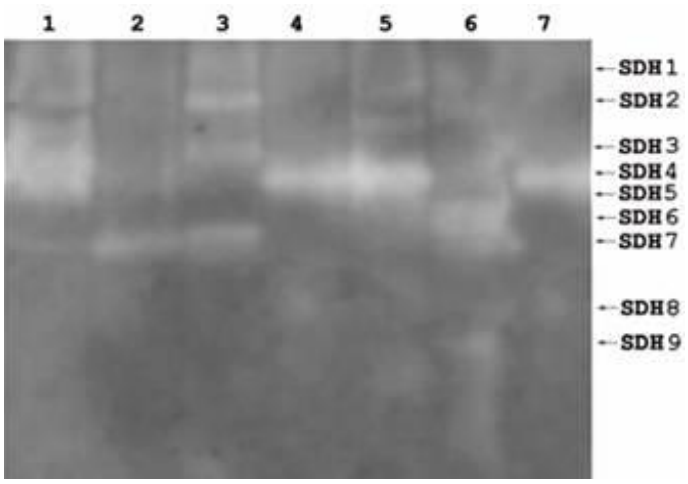

Figure 10. Succinate dehydrogenase isozyme profile of *Foc* and *Fo* isolates. The numbers represent the isolates.

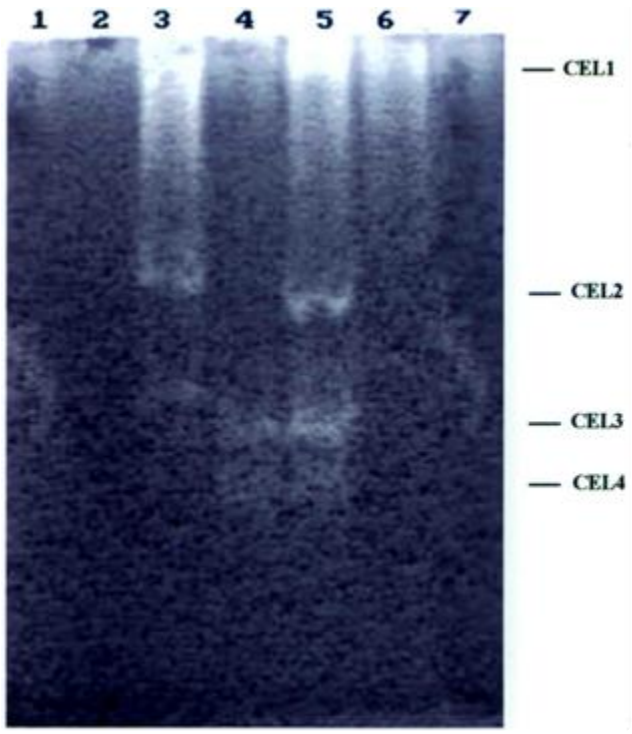

Figure 9. Cellulase isozyme profile of *Foc* and *Fo* isolates. The numbers represent the isolates.

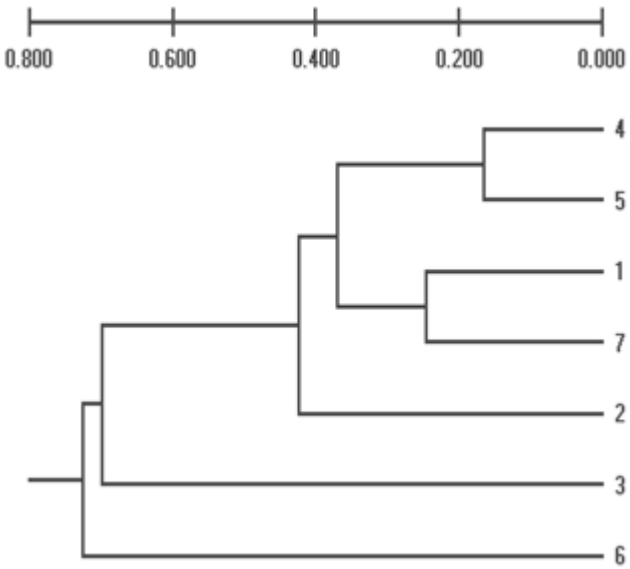

Figure 11. Dendrogram showing relationship among the six isolates of *F. oxysporum* f. sp. *cubense* isolates and one isolate of *F. oxysporum*.

selection for resistant source. The use of isozyme markers for estimating the extent of diversity within a fungal population is well documented (Micales et al., 1986; McDonald and McDermott, 1993); biological and genetic characteristics of the Foc isolates colonizing banana cultivar 'Nanjangud rasabale' are closely related. All the isolates showed one or two similar bands for each enzyme tested and this confirms that all isolates are Foc and it was confirmed by the pathogenicity test.

VCG test showed clear compatibility between isolates 1, 2, 3, 4, 5 and 7. Isolate 6 was not compatible with any other isolates (data not shown). If two different strains of a fungus are unable to form a heterokaryon when they are paired, they are said to be vegetatively incompatible. Two strains that form a heterokaryon are vegetatively compatible (Puhalla, 1985). All mutually compatible isolates were assigned to one group, called vegetative compatibility group, or VCG. Although the sample was small, there was correlation between VCG and pathogenicity. Earlier investigations support this view in *F. oxysporum* f. sp. *apii* (Puhalla and Spieth, 1983) and in *Verticillium dahliae* (Puhalla and Hummel, 1983).

The biochemical and molecular markers are being used as versatile and highly informative tools for identifications and diagnosis of fungal pathogens (Majer et al., 1996) and for population studies (McDonald and McDermott, 1993; McDonald et al., 1999). They can be used to evaluate levels of genetic diversity and phenotypic relationships within and between species and to identify races and pathotypes (Brown, 1996). The electrophoretic separation of protein, including isozymes, is a useful tool for differentiating fungal taxa. Bosland and Williams (1987) have used isozyme to differentiate *Fusarium* species and four *F. oxysporum* formae specials, as well as isolates obtained from different crucifers species. On the contrary, no clear-cut differences were observed for isozymes of *F. oxysporum* f. sp. *lycopersici* recovered from different sites or vegetative compatibility groups (Elias and Schneider, 1992). SDS-PAGE is used because the methods alleviated the need for culturing and samples are analyzed in a more direct manner. The results obtained by SDS-PAGE of whole-cell proteins can discriminate almost at same level as DNA fingerprinting (Priest and Austin, 1993) in some cases.

The SDS-PAGE technique used for analyzing the total proteins from the isolates of Foc is relatively simple and inexpensive for differentiation and identification of isolates and has been used previously for studying variation in a number of fungal populations (Burgess et al., 1995; Aly et al., 2003). Many of the common bands observed between isolates suggested that they are more closely related. Identification of a protein of molecular mass 43.0 and 51.9 kDa was unique to isolate 1. The low genetic variability found in Foc isolates found in the present study could be due to asexual mode of reproduction. These results agree with those obtained by Belisario et al. (1998), as no differences were found among mycelium protein profiles (SDS-PAGE) of different species and formae specialis of *F. oxysporum*, *F. solani* and *F. culmorum*.

In the 1960s and 1970s, analytical techniques were developed for utilizing isozyme data for numerical taxonomy. Because the majority of animal and plant populations for which these techniques were developed were diploid, sexually reproduced species, several of the assumptions that must be met to apply these techniques were not applicable to haploid, asexual organisms such as *F. oxysporum*. Although multiple bands for a given enzyme may result from post-translational modification of multiple subunits, as well as different alleles to loci, sexual crosses that would clarify these relationships are not possible with this fungus. Thus, bands can only be treated as putative loci and alleles. When they are analyzed as phenotypic characters, it is possible to quantitatively assess genetic diversity within *F. oxysporum*. The relatedness of many fungal taxa has been assayed by using isozymes (Huss et al., 1996; Banke et al., 1997).

In general, the results indicated polymorphism for the enzymes investigated. Burdon (1993) observed uniformmity of isozyme markers and also considerable variation in some other genetic markers like virulence. In the present study, no differences were found among Foc isolates as far as physiological (pathogenicity) and phenotypic (morphology and growth) characteristics, but the biochemical characterization correlated with the data obtained by RAPD and IGS analysis (data not shown) using cluster analysis with Unweighted Pair-Group Method with arithmetic mean (UPGMA) using genetic distances.

The isozyme patterns may provide additional information about the genetic structure of *Foc*. Unambiguous interpretation requires genetic crosses to determine the mode of inheritance of these markers. The polymorphic isozyme systems could serve as an indicator of genetic variability in Foc and in identifying and characterizing Foc isolates.

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